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(54) **OSTEOINDUCTIVE PROTEIN MIXTURES AND PURIFICATION PROCESSES**

OSTEOINDUKTIVE PROTEINMISCHUNGEN UND REINIGUNGSVERFAHREN

MELANGES DE PROTEINES OSTEOINDUCTIVES ET PROCEDES DE PURIFICATION

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Description

Field of the Invention

[0001] The present invention relates generally to proteins useful in inducing or promoting bone growth (i.e. osteoinductive proteins) and to processes used to purify such proteins from extracts of demineralized bone. More specifically, the invention relates to an ultrafiltration process, an anion exchange process, a cation exchange process, and a reverse phase high performance liquid chromatography (HPLC) process, preferably employed in combination, to purify bone-derived proteins.

Background of the Invention

[0002] Bones include many proteins, some of which induce or promote bone growth. A great deal of research has been directed to producing, either by recombinant DNA techniques or by purification of naturally occurring proteins, specific osteoinductive proteins. Such proteins and a variety of processes for obtaining them are the subject of numerous patents. However, very little work has been directed to the economic, large scale commercial production of useful osteoinductive proteins.

[0003] Collagen Corporation of Palo Alto, California is the assignee of a number of patents directed to osteoinductive proteins. U.S. Patent No. 4,434,094 by Seyedin et al., issued February 28, 1984 identifies a process to partially purify an osteogenic factor and isolate a non-fibrous protein having a molecular weight less than 30 kilodaltons (kD) from demineralized bone extract using cation exchange chromatography. A partially purified bone-inducing factor of 10 to 30 kD and the purification process including extraction from demineralized bone, gel filtration, and cation exchange chromatography on a carboxymethyl cellulose column, and which may include reverse phase-high performance liquid chromatography (HPLC), is described in U.S. Patent No. 4,627,982 by Seyedin et al., issued December 9, 1986. Also by Seyedin et al. and assigned to Collagen Corp., U.S. Patent No. 4,774,228 issued September 27, 1988, describes two 26 kD proteins found in bone having activity in a TGF-β assay and purified using a process similar to that taught in Seyedin's '094 patent but including reverse phase HPLC or acetic acid-urea gel electrophoresis, where the purified proteins exhibit chondrogenic activity (purportedly related to bone formation). U.S. Patent No. 4,863,732 by Nathan et al., issued September 5, 1989 is directed to an injectable solution of an osteogenic factor such as that described in Seyedin's '982 patent, combined with atelopeptide collagen and further purified by coprecipitation. Other patents relate to mixtures of atelopeptide collagen material, e.g. U.S. Patent Nos. 4,789,663 by Wallace et al., issued December 6, 1988 and 4,795,467 by Piez et al. issued January 3, 1989.

[0004] Marshall R. Urist is an inventor named in numerous patents in the field of bone inducing agents. U.S. Patent No. 4,294,753 by Urist, issued October 13, 1981, describes a process for obtaining bone morphogenetic protein (BMP) by treating demineralized bone with a neutral salt to transform the bone collagen to gelatin, extracting the BMP with a solubilizing agent, then removing the solubilizing agent and salt by dialysis to precipitate the BMP. It is recognized that precipitation of proteins from solution is not highly selective. The BMP, with a molecular weight ranging from 1,000 to 100,000 is the subject of U.S. Patent No. 4,455,256 by Urist, issued June 19, 1984. U.S. Patent No.4,619,989 by Urist, issued October 28, 1986, discloses an improved process for further purifying and isolating human and bovine BMP compositions and factors, including additional dialysis and co-precipitation steps. U.S. Patent No. 4,761,471 by Urist issued August 2, 1988 relates to products obtained by the aforementioned process including a substantially pure BMP composition containing an active 17.5 kD (human) or 18.5 kD (bovine) BMP factor and BMP associated proteins with molecular weights of approximately 14, 22, 24, and 34 kD which may enhance but do not induce bone formation.

[0005] U.S. Patent No. 4,877,864 by Wang, et al., issued October 31, 1989 discloses human and bovine bone inductive factors of approximately 28 kD to 30 kD and characterized by a specific peptide sequence, which may be produced by recombinant gene techniques.

[0006] U.S. Patent No. 4,804,744 by Sen, issued February 14, 1989 identifies a primary osteogenic protein (P_3) with a molecular weight of about 22 to 24 kD. This patent also identifies proteins P_2 and P_4 which are nonosteogenic without P_3, and further identifies a method for isolating P_3 from demineralized bone tissue including extractions, dialysis, gel filtration and HPLC steps.

[0007] As is apparent, it would be desirable to have a mixture of proteins which are highly osteoinductively active. It would be beneficial if such proteins could be produced efficiently and effectively on a commercial scale. It would also be beneficial if such proteins could be produced in a manner designed to minimize degradation of such proteins while maximizing production. It would also be beneficial if such proteins could be produced using relatively well-known unit operations in a process which is tolerant of minor variations in process conditions. It would also be beneficial if the mixture of proteins could be produced directly, without having to first obtain single specific proteins and remix them to attain the desired mixture.

Summary of the Invention

[0008] The present invention includes a process for obtaining an osteoinductive factor, preferably by purification from bovine bone, and the resulting product.

[0009] One embodiment of the process for purifying osteoinductively active proteins comprises conducting anion exchange chromatography on a demineralized bone extract solution, preferably having a pH from about pH 8 to about pH 9 and preferably having a conductivity below about 1,900 μmhos (1.9×10^{-3} S). Proteins are eluted from the anion exchange resin with an eluant, preferably having a conductivity from about 10,260 μmhos (1.026×10^{-2} S) to about 11,200 μmhos (1.120×10^{-2} S). The solution of eluted proteins is preferably adjusted to a pH from about pH 4.4 to about pH 5.0 and preferably to a conductivity from about 17,900 μmhos (1.79×10^{-2} S) to 19,200 μmhos (1.92×10^{-2} S) and is loaded onto cation exchange resin. Proteins are eluted from the cation exchange resin with an eluant, preferably having a conductivity from about 39,100 μmhos (3.91×10^{-2} S) to about 82,700 μmhos (8.27×10^{-2} S). Conductivity values higher than 82,700 μmhos can also be successfully employed, however, higher values would require a longer time period to dialyze prior to HPLC. The proteins eluted from the cation exchange resin are loaded onto a reverse phase HPLC column. Proteins are eluted from the HPLC column with an eluant having an increasing acetonitrile concentration gradient, preferably ranging from about 33 percent by volume to 37 percent by volume, to obtain a purified mixture of proteins having enhanced osteoinductive activity.

[0010] In a further embodiment of the invention, the anion exchange resin is strongly positive and has quaternary amine functional groups. In another embodiment of the invention, the cation exchange resin is strongly negative and has sulfonic acid functional groups. The invention also includes the use of a HPLC packing material which is a hydrocarbon-modified silica and preferably, is a VYDAC™ (The Separation Group) C_{18} column.

[0011] The invention also includes an osteoinductive factor obtained by the above-described process. In one embodiment of the osteoinductive factor, the factor is a mixture of a number of proteins having the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile shown in Figure 1. Another embodiment of the present invention is a mixture of proteins having a preferred amino acid composition of about 23.4 mole percent of acidic amino acids [ASP(+ASN) and GLU(+GLN)]; about 13.5 mole percent of hydroxy amino acids (SER and THR) about 40.0 mole percent aliphatic amino acids (ALA, GLY, PRO, MET, VAL, ILE, and LEU); about 6.8 mole percent aromatic amino acids (TYR and PHE); and about 16.6 mole percent basic amino acids (HIS, ARG and LYS). TRP, CYS and ½ CYS were not measured and are not included in the calculation of mole percent.

[0012] In accordance with a preferred embodiment of the present invention, demineralized bone particles are subjected to protein extraction using guanidine hydrochloride. The extract solution is filtered, and subjected to a two step ultrafiltration process. In the first ultrafiltration step an ultrafiltration membrane having a nominal molecular weight cut off (MWCO) of 100 kD is preferably employed. The retentate is discarded and the filtrate is subjected to a second ultrafiltration step using an ultrafiltration membrane preferably having a nominal MWCO of about 10 kD. The retentate is then subjected to diafiltration to substitute urea for guanidine. The protein-containing urea solution is then subjected to sequential ion exchange chromatography, first anion exchange chromatography followed by cation exchange chromatography. In the process described above, the osteoinductively active proteins are advantageously kept in solution. Preferably, the osteoinductive proteins produced by the above process are then subjected to HPLC.

[0013] An advantage of the present invention is that a process is provided which can be readily scaled up to a commercial production scale. A further advantage is that the proteins are kept in solution during the purification steps. Another advantage is that the proteins exhibit little deterioration during the production process. Another advantage is that, if desired, the resultant mixture of proteins can be used directly, without remixing with proteins produced by separate processes.

Brief Description of the Drawings

[0014]

Fig. 1 illustrates an SDS-PAGE of the osteoinductively active protein mixture, both in reduced and nonreduced forms, obtained in accordance with the process of the present invention; and

Fig. 2 illustrates the x-ray standards employed to obtain the "X-Ray Score" employed in the Examples.

Detailed Description of the Invention

[0015] In accordance with the present invention, a process for purifying an osteoinductive factor from bone is provided and an osteoinductive factor is provided.

[0016] In one embodiment of the invention, the process for purifying bone derived proteins includes an ultrafiltration step, an anion exchange chromatography step, and a cation exchange chromatography step. Other embodiments of

the invention include an HPLC purification step.

[0017] Another aspect of the invention is an osteoinductive mixture of proteins. In one embodiment, the mixture includes several proteins. This is illustrated in Fig. 1 which shows an SDS-PAGE of the reduced and non-reduced osteoinductive factor. In another embodiment the invention also includes an osteoinductively active mixture of proteins with an amino acid composition of about 20.7 to about 26.1 (preferably about 23.4) mole percent of ASP (+ASN) and GLU(+GLN); about 11.3 to about 15.7 (preferably about 13.5) mole percent SER and THR; about 37.6 to about 42.4 (preferably about 40.0) mole percent ALA, GLY, PRO, VAL, MET, ILE, and LEU; about 5.8 to about 7.9 (preferably about 6.8) mole percent TYR and PHE; and about 13.3 to about 19.9 (preferably about 16.6) mole percent HIS, ARG, and LYS. Further embodiments include the osteoinductive factors resulting from each embodiment of the described purification process.

[0018] An "osteoinductive factor" as used herein refers to a composition containing one or more proteins which is osteoinductively active as determined by a histological evaluation showing the de novo formation of bone with accompanying osteoblasts, osteoclasts, and osteoid matrix. For example, osteoinductive activity can be demonstrated by a test using a substrate onto which material to be tested is deposited. A substrate with deposited material is implanted subcutaneously in a test animal. The implant is subsequently removed and examined microscopically for the presence of bone formation including the presence of osteoblasts, osteoclasts, and osteoid matrix.

[0019] A preferred starting material for the present process is obtained by a multi-step procedure which preferably includes preparing the bone for extraction of proteins by grinding, cleaning, and demineralizing the bone; extracting the bone proteins; and concentrating the extracted proteins with multiple purification steps. The preferred source of starting material for the present process is mammalian bone. Because of its ready availability and low costs, bovine bone is typically used. Other mammalian bone, however, can be suitable for practicing the invention. The bone is prepared for extraction of bone proteins by customary means known in the art, such as grinding and cleaning the bone. Typically, the bone is ground into successively finer particles and soaked in detergent solution to remove non-bone material. Preferably the bone is ground to particles less than 4 mm in size and preferably from about 1 mm to about 4 mm in size, soaked in detergent solution between grindings, and rinsed in a flotation tank to remove soft tissue.

[0020] The cleaned ground bone is then demineralized with acid. The bone is soaked in any suitable acid and may be agitated, preferably at room temperature. The pH of the acid soaking solution typically is maintained at or below pH 1.3. A solution of dilute HCl (e.g. from about 0.6 M to about 1.2 M) has proven effective to demineralize bone. Alternatively other suitable acids such as formic acid can be used. Octyl alcohol or other defoaming agents are useful to prevent excessive foaming during demineralization.

[0021] The bone is soaked in acid for sufficient time until the bone is substantially completely demineralized. X-ray analysis may be used to evaluate the extent of demineralization. Alternatively, standard procedures can be developed through experience to determine the amount of time required for demineralization. Typically, at least seven hours is required. The demineralized bone is then rinsed to remove the acid. Typically the bone is rinsed with water overnight or until the pH of the rinse discharge reaches pH 4 or more. As will be appreciated by those skilled in the art, alternative cleaning and demineralizing processes can also be employed.

[0022] Proteins are extracted from the demineralized bone using a protein denaturant such as guanidinium ion and/or urea. Preferably the extraction is performed at less than 20°C and more preferably at less than 15°C. It should be noted that other suitable denaturants can be used as well. Guanidine hydrochloride is a preferred denaturant because it is ionic and therefore also functions well as a solubilizing agent for maintaining proteins in solution.

[0023] Optionally, a chaotrope can be added during extraction to improve solubility of extracted proteins. Suitable chaotropes include calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), and cesium chloride ($CeCl_2$).

[0024] Protein can be extracted from demineralized bone by means typically used in the art. For example, a protein denaturant can be pumped through demineralized bone in a filter press to extract proteins in recovered denaturant. In order to provide appropriate low temperatures, the denaturant can be cooled to an initially low temperature, preferably from about 0°C to about 4°C, as it is pumped through the demineralized bone. The temperature of the denaturant can increase during the extraction process.

[0025] Usually, extraction continues until substantially all of the noncollagenous bone proteins have been removed from the demineralized bone. A typical extraction takes about 48 hours. Preferably the extraction solution is maintained near neutral pH.

[0026] The extracted proteins in a denaturant solution are separated by a series of purification steps. A first ultrafiltration process selects for desired proteins with a molecular weight within a preselected, desired range for further processing. Preferably, as a first step, an ultrafiltration membrane with a 100 kD nominal molecular weight cutoff (MWCO) is used, such as a plate and frame tangential flow filtration unit sold under the trade name "Centrasette"™ (Filtron). The filtration is preferably conducted under pressure and typically at about 50 psi filtration pressure. The protein concentration will vary depending on the completeness of the extraction.

[0027] Following the first ultrafiltration step, the filtrate is concentrated by a second ultrafiltration step, preferably across a 10 kD nominal molecular weight cutoff membrane, which eliminates lower molecular weight proteins. The

second ultrafiltration yields a retentate with a mixture of proteins having molecular weights within a desired range ("filtered protein concentrate").

[0028] In a preferred embodiment, in preparation for subsequent ion exchange chromatography, the filtered protein concentrate is transferred from an ionic denaturant solution to a non-ionic denaturant solution, such as urea. A non-ionic denaturant is preferred for use in the subsequent ion exchange purification steps because an ionic denaturant, such as guanidine hydrochloride, impairs the ability of the ion exchange resins to selectively bind desired proteins. Preferably, the protein denaturant solution is from about 2 M to about 6 M urea solution which is buffered with tris [hydroxymethyl]aminomethane (hereinafter referred to as "tris") and titrated to pH of about pH 8.5.

[0029] The transfer of proteins from an ionic to a non-ionic denaturant can be accomplished using diafiltration or dialysis. Diafiltration is performed using a suitable tangential flow ultra-filtration unit such as a Centrasette™. (Filtron) ultrafiltration unit. Through the use of diafiltration or dialysis the filtered protein concentrate can be transferred to the appropriate denaturant without precipitation of proteins from solution. This procedure is advantageous because it simplifies subsequent purification and prevents losses that can occur if proteins are allowed to precipitate. It is also advantageous that this procedure can be used on a commercial production scale.

[0030] The present invention includes an anion exchange process to purify a solution of extracted bone proteins to yield an osteoinductively active mixture of proteins. In a preferred embodiment of the anion exchange process, the starting material is prepared using the above-described process for obtaining a preferred starting material. The anion exchange process is combined with additional purification processes described below.

[0031] In ion exchange chromatography, i.e., anion exchange and cation exchange chromatography, the affinity of a particular protein for a particular ion exchange resin depends on the ionic strength of the protein solution and pH. The ionic strength of a solution can be measured by its conductivity. Alternatively, the ionic strength of a solution can be measured in terms of a specific counterion concentration. As used herein, the term "counterion concentration" refers to the molar concentration of an ion in solution which competes with proteins for binding sites on the ion exchange resin.

[0032] Prior to loading a bone protein solution onto an anion exchange column in accordance with the present invention, the conductivity of the bone protein solution is adjusted to allow the desired proteins to bind selectively to the resin. In the present anion exchange process, the conductivity of the bone protein solution is adjusted to less than about 1,900 μmhos (1.9×10^{-3} S), and more preferably from about 1,080 μmhos (1.08×10^{-3} S) to about 1,900 μmhos (1.9×10^{-3} S) by variation in the counterion concentration. In a preferred embodiment, the counterion is Cl^- and is present in a concentration of less than about 0.135 M NaCl.

[0033] The bone protein solution to be purified by the anion exchange process; having an appropriate conductivity, is loaded onto an anion exchange column. In the present anion exchange process, the anion exchange column has a strongly positive anion exchange resin. It has been found that the present anion exchange process with a strongly positive anion exchange resin is effective in purifying an osteoinductively active mixture of proteins. As used herein, the term strongly positive anion exchange resin refers to a resin having strongly positive functional groups such as quaternary amine functional groups. A preferred resin with quaternary amine functional groups is sold under the trademark "Q-Sepharose"™ (Pharmacia). However, other resins having similarly basic functional groups are suitable as well.

[0034] A further factor affecting the selectivity of binding to the anion exchange resin is the pH of the bone protein solution being loaded onto the anion exchange column and the anion exchange eluant. The pH of the bone protein solution and eluant is at a pH effective to allow the resin to bind desired proteins from the bone protein solution as it passes through the column and to allow for desired elution of proteins. Generally, a pH of between about pH 8 and pH 9 is effective in the present process. Preferably, the pH of the protein solution and eluant is adjusted to about pH 8.5.

[0035] The linear velocity of the bone protein solution through the anion exchange column is determined by required recovery parameters. Typically, the process is run at a low velocity to allow for substantially complete adsorption of desired proteins so that protein loss is minimized. It should be recognized, however, that the linear velocity can be greater, but that protein loss may be sustained.

[0036] The anion exchange process further includes selectively desorbing a desired fraction of bound proteins from the column resin with an eluant. The fraction of bound proteins which are desorbed is determined by the conductivity of the eluant solution. Proteins are eluted from the anion exchange column of the present invention with a solution having a conductivity sufficient to obtain an osteoinductively active protein mixture. Preferably, the eluant has a conductivity from about 10,260 μmhos (1.026×10^{-2} S) to about 11,200 μmhos (1.120×10^{-2} S). Higher conductivities may be employed, however, this leads to the desorption of a greater amount of materials which may have to be removed during a subsequent purification step.

[0037] The anion exchange eluant in the present invention is typically a protein denaturant solution having an appropriate salt concentration to obtain the appropriate conductivity. A preferred eluant is 6 M urea buffered with tris base containing sodium chloride. Sodium chloride (NaCl) is effective to provide a counterion concentration which yields the appropriate conductivity in the eluant. In a preferred embodiment, the eluant is prepared with a counterion concentration of NaCl between about 0.10 M and about 0.16 M and more preferably between about 0.105 M and about 0.145 M. Other suitable salts may also be used at a counterion concentration sufficient to provide the appropriate conductivity.

[0038] The present invention further includes a cation exchange chromatography process to further purify the osteoinductive factor from bone-derived proteins in solution which may be used advantageously in combination with the anion exchange process described above and the HPLC process described below.

[0039] As discussed above with respect to the anion exchange process, the conductivity of the bone protein solution is controlled to effect selective binding of proteins to the cation exchange resin. Prior to loading the bone protein solution onto a cation exchange column in the present invention, the conductivity of the bone protein solution is adjusted to be effective to allow the cation exchange resin to selectively bind a desired fraction of proteins. In the present cation exchange process, the conductivity of the bone protein solution is preferably from about 17,900 µmhos (1.79×10^{-2} S) to about 19,200 µmhos (1.92×10^{-2} S). Sodium chloride or other suitable salt can be used to adjust the conductivity to an appropriate level. In a preferred embodiment, the counterion concentration of the bone protein solution is between about 0.125 M NaCl and about 0.30 M NaCl, and more preferably between about 0.23 M and about 0.27 M NaCl.

[0040] The bone protein solution to be purified by the cation exchange process, having an appropriate conductivity, is loaded onto a cation exchange column. In the present cation exchange process, a strongly negative cation exchange resin has been found effective in purifying a mixture of osteoinductively active proteins. As used herein, the term strongly negative cation exchange resin refers to a resin having strongly negative functional groups such as sulfonic acid functional groups. A preferred resin with sulfonic acid functional groups is sold under the trademark "S-Sepharose"™ (Pharmacia). However, other resins having similarly acidic functional groups are suitable as well.

[0041] The pH of the bone protein solution to be purified by the cation exchange process is adjusted to a pH effective for the binding of desired proteins to the resin. In the present process, a pH of between about pH 4.4 and about pH 5.0 is preferably used. Preferably the pH of the protein solution is adjusted to about pH 4.8.

[0042] The linear velocity of the bone protein solution through the cation exchange column, as with the anion exchange above, is determined by required recovery parameters. The velocity is generally sufficiently low to permit substantially complete adsorption of the desired proteins with minimal protein loss.

[0043] The cation exchange process further includes selectively desorbing a desired fraction of bound proteins from the column resin. Proteins are eluted from the cation exchange column with an eluant having a conductivity suitable to yield an osteoinductively active protein mixture. For the present cation exchange process, the conductivity of the eluant is preferably from 39,100 µmhos (3.91×10^{-2} S) to about 82,700 µmhos (8.27×10^{-2} S) or more.

[0044] Generally, the eluant for the present process is a solution having a suitable protein denaturant, such as urea, and an appropriate salt concentration to achieve the desired conductivity. In a preferred embodiment, the eluant is prepared with a counterion concentration from about 0.6 M NaCl to about 1.5 M NaCl and more preferably from about 1.3 M to about 1.5 M NaCl to provide the appropriate conductivity.

[0045] The present invention further includes a reverse phase HPLC process which may be combined with the anion and cation exchange processes described above to obtain an osteoinductively active mixture of proteins. In the HPLC purification process of the present invention, a bone protein solution is loaded onto a reverse phase HPLC column. This column may be polymeric (i.e., polystyrene) or silica based. Preferably, the HPLC column is a hydrocarbon modified silica. Preferably, a silica resin is modified by the addition of C_4 - C_{18} hydrocarbon chains, and more preferably, the HPLC column is a VYDAC™ (The Separation Group) C_{18} column.

[0046] The bone protein solution to be loaded onto the reversed phase column can be a solution of trifluoroacetic acid or other suitable solvent (e.g. heptafluorobutyric acid or phosphoric acid). Preferably, a trifluoroacetic acid solution is used having a concentration of from about 0.05 percent by volume to about 0.15 percent by volume, and more preferably about 0.1 percent by volume trifluoroacetic acid.

[0047] Osteoinductively active proteins are eluted from the HPLC column with an organic solvent/water mixture suitable for obtaining the desired proteins. A preferred eluant in the HPLC process is an acetonitrile solution. The preferred eluant typically has an acetonitrile concentration which varies, during elution, from about 30 percent by volume to about 40 percent by volume and more preferably from about 33 percent by volume to about 37 percent by volume. In preferred embodiments, the acetonitrile concentration in the eluant is increased in increments of between about 0.30 percent by volume and about 0.40 percent by volume per minute until the desired highest concentration of acetonitrile is achieved. Proteins can be recovered from the HPLC process eluant by means generally known in the art.

[0048] A further embodiment of the present invention is the protein product from the above-described HPLC process which exhibits osteoinductive activity at about 3 micrograms when deposited onto a suitable carrier and implanted subcutaneously. In one embodiment of the invention, the osteoinductive factor is an osteoinductively active mixture of proteins which exhibit the gel separation profile shown in Figure 1. This gel separation profile was performed using SDS-PAGE. The first column is a molecular weight scale which was obtained by performing SDS-PAGE on standards of known molecular weight. The second column illustrates the SDS-PAGE profile for a mixture of proteins in accordance with the present invention which have been reduced with 2-mercaptoethanol. The third column illustrates the SDS-PAGE profile for a non-reduced mixture of proteins in accordance with the present invention. Although the mixture of proteins which provide the SDS-PAGE profile illustrated in Figure 1 have been found to have high osteoinductive activity, as will be demonstrated in the examples, it is anticipated that mixtures of proteins having SDS-PAGE profiles

which differ slightly from that illustrated in Figure 1 will also be effective. Therefore, mixtures of proteins having profiles which comprise substantially all of the protein bands detected in the reduced or nonreduced SDS-PAGE profiles in Figure 1 will be considered to be within the scope of the invention.

[0049] Yet another embodiment of the invention includes an osteoinductively active mixture of proteins having, upon hydrolysis, an amino acid composition of about 23.4 mole percent of ASP(+ASN) and GLU(+GLN); about 13.5 mole percent SER and THR; about 40.0 mole percent ALA, GLY, PRO, MET, VAL, ILE, and LEU; about 6.8 mole percent TYR and PHE; and about 16.6 mole percent HIS, ARG and LYS.

[0050] An osteoinductively active mixture of proteins, as derived by any of the above-described processes, or by some other process, can be delivered to a site where bone growth is desired using a variety of delivery systems. One delivery system is a collagen substrate on which an osteoinductively active mixture of proteins is deposited. A further embodiment of the invention is a delivery system using skin-derived or tendon-derived collagen.

EXAMPLES

Example 1

[0051] Bovine cortical bone segments (47 kg) were ground through successive screens of 25, 6 and 4 mm pore size. After each grinding, the bone particles were cleaned in a flotation tank containing a detergent solution to facilitate soft tissue and lipid removal. The ground bone (25.95 kg) was demineralized by stirring with 60 gallons (227 liters) of a 1.2 M HCl solution for 7.5 hours at room temperature. Forty milliliters of octanol was also added to prevent foaming.

[0052] Osteoinductively active proteins were extracted with approximately 60 l of 4 M guanidine hydrochloride, buffered with tris base to pH 7.4, by continuously circulating the solution through a packed bed of the demineralized bone particles. The guanidine was initially cooled to about 4°C during the extraction, in which 59 liters of extract solution was collected after 49.5 hours.

[0053] The extract solution was filtered through a 5 µm capsule filter then concentrated to a volume of 700 ml using 25 sq. ft. (2.32 square meters) of Filtron Omega™ tangential flow ultrafiltration membrane with a nominal molecular weight cut-off (MWCO) of 100 kD. The retentate was discarded, and the less than 100 kD MW filtrate was concentrated to a volume of 1.1 l on 25 sq. ft. (2.32 square meters) of Filtron Omega™ ultrafiltration membrane with a nominal MWCO of 10 kD. Thirty 1 of 20 mM tris and 6 M urea, adjusted to pH 8.5 with HC1, was then used to diafilter the solution using the same 10 kD MWCO membrane. The 950 ml of retentate contained approximately 19.27 grams of protein. The filtrate was discarded.

[0054] The retentate was loaded onto a Q-Sepharose™ (Pharmacia) anion exchange column (25.2 cm diameter x 16.5 cm, bed volume = 8.3 1) equilibrated with 20 mM tris and 6 M urea, adjusted to pH 8.5 with HCl (conductivity = 910 µmhos; 9.1×10^{-4} S). Following sample application, the column was washed with approximately 2 void volumes of equilibration buffer. Osteoinductively active proteins were eluted from the column by applying approximately 12 1 of 0.125 M NaCl in 20 mM tris and 6 M urea, adjusted to pH 8.5 (conductivity = 10,740 µmhos; 1.074×10^{-2} S). Approximately 3.94 g of protein in 11 l were recovered in the column eluate.

[0055] Eluate from Q-Sepharose™ (Pharmacia) anion exchange chromatography was adjusted to pH 4.8 with glacial acetic acid and the conductivity was increased to 18,200 µmhos (1.82×10^{-2} S) by adding solid NaCl. The sample was then loaded onto an S-Sepharose™ (Pharmacia) cation exchange column (11.3 cm diameter x 16.5 cm, bed volume = 1:65 l) equilibrated with 0.25 M NaCl in 20 mM sodium acetate and 6 M urea, adjusted to pH 4.5. Following sample application, the column was washed with approximately 2 void volumes of equilibration buffer. Osteoinductively active proteins were eluted by applying approximately 1.7 l of 1.5 M NaCl in 20 mM sodium acetate and 6 M urea, adjusted to pH 4.5 (conductivity = 79,500 µmhos; 7.95×10^{-2} S). Approximately 220.8 mg of protein in 1.2 l were recovered in the column eluate.

[0056] The cation exchange eluate was dialyzed in the cold (4°C) against deionized water using a 6 kD molecular weight cut off hollow fiber bundle (obtained from Spectrum Industries), in order to remove low molecular weight species, and freeze-dried. It was redissolved in 10 percent (v/v) acetic acid, freeze-dried, then dissolved again in 10 mM HCl. After sequential filtration through 1.2 and 0.45 µm filters, the protein mixture was allowed to stand at room temperature for 18 to 24 hours then freeze-dried.

[0057] By the series of steps outlined here, the osteoinductively active proteins were maintained in solution. Protease inhibitors were not used at any step, and no degradation of the proteins was observed. Following cation exchange chromatography and removal of salts and buffers, 35 µg of the proteins isolated by this procedure reproducibly induced bone formation when combined with a suitable carrier or matrix and implanted subcutaneously in rats.

Example 2

[0058] The freeze-dried sample from S-Sepharose™ (Pharmacia) cation exchange chromatography was dissolved

in 6 ml of a mixture of aqueous 0.1 volume percent trifluoroacetic acid/30 volume percent acetonitrile and applied to a preparative VYDAC™ (The Separation Group) C_{18} wide pore HPLC column equilibrated with 57% A, 43%B (where A is 0.1 volume percent trifluoroacetic acid in water and B is 70 volume percent acetonitrile, 0.1 volume percent trifluoroacetic acid in water). The osteoinductively active proteins were separated from other components of the sample using a shallow gradient of increasing B. Eluate from the HPLC was characterized by SDS-polyacrylamide gel electrophoresis and in vivo bioassay. Osteoinductively active proteins were found to elute between 47 volume percent B and 52 volume percent B (33 percent to 37 percent acetonitrile by volume). Proteins eluting over this range of solvent composition were freeze-dried, then dissolved in 10 mM HCl. The yield of osteoinductively active proteins was 16.45 mg. When deposited on a suitable carrier or matrix and implanted subcutaneously, 3.5 µg of this protein mixture induced bone formation.

Example 3

[0059] There are a number of characteristic protein bands which are present in the osteoinductively active pool. They range between 14 kD and 68 kD apparent MW based on electrophoretic migration in 15 weight percent SDS-polyacrylamide gels. Examples of the protein banding pattern before and after reduction with 2-mercaptoethanol are illustrated in Figure 1.

Example 4

[0060] Aliquots from the osteoinductively active pool from preparative HPLC were hydrolyzed in 6 M HCl vapor at 110°C for 20 hours under an argon atmosphere. Hydrolyzed samples were derivatized with phenylisothiocyanate to form the PTC-amino acid derivatives, and analyzed by reversed phase HPLC using a Beckman 338 gradient system equipped with System Gold software. Three amino acid analyses were averaged to establish the composition presented in Table I.

TABLE I

Amino Acid	Mole Percent
Asp	11.14
Glu	12.25
Ser	9.48
Gly	8.50
His	2.28
Arg	7.19
Thr	4.03
Ala	8.05
Pro	7.16
Tyr	3.63
Val	3.79
Met	1.73
Ile	2.75
Leu	8.00
Phe	3.21
Lys	7.11

Example 5

Evaluation of the osteoinductive activity of purified and partially purified osteoinductive factor.

[0061] A sufficient quantity of purified Type I fibrillar bovine tendon collagen was added to a 1 volume percent solution

of acetic acid in water to make a 4 weight percent dispersion. After standing overnight at room temperature, the viscous dispersion was placed into a multicavity DELRIN™ (DuPont) mold making discs 8 mm in diameter x 3 mm thick. The mold of collagen dispersion was frozen at -50°C and lyophilized for about eighteen hours yielding discs of collagen sponge weighing 6.0 ± 1 mg each. These discs served as the substrate to which the osteoinductive factor was added during the in vivo biological evaluation of osteoinductive activity.

PURIFIED OSTEOINDUCTIVE FACTOR

[0062] To a 1 x 10^{-2} M solution of hydrochloric acid was added a sufficient quantity of purified osteoinductive factor to prepare test solutions containing 35,100 and 350 µg of protein per ml HCl. One hundred µl aliquots of the above three test solutions were added to four collagen sponge discs for each test dose. The solution was allowed to soak into the collagen sponge discs for thirty minutes whereupon the discs were frozen at -50°C and lyophilized for about eighteen hours. These purified osteoinductive factor-containing collagen sponge discs were implanted subcutaneously in four rats in a manner similar to that described by Reddi (Reddi, A.H., "Regulation of Bone Differentiation by Local and Systemic Factors" in Bone & Mineral Research, Vol. 3, Chap. 2, ed. William Peck (Elsevier Publishers B.V., 1985) and as described below.

[0063] A small (≈6 mm) incision was made in the skin of the ventral thorax region of a female Long-Evans rat, weighing approximately 50 to 100 g. A pocket was prepared beneath the skin by blunt dissection. One of the previously prepared collagen sponge discs containing purified osteoinductively active protein was inserted in the pocket and the incision was closed with Tevdek II™ (Ethicon) 5-0 sutures. One each of the other two purified osteoinductively active protein dose group samples were similarly implanted in each animal. The implanted collagen sponge discs were separated from each other by a minimum of 1 cm distance. After 21 days, the rats were sacrificed by asphyxiation with carbon dioxide and the test materials were removed. At explantion, the tissue samples were weighed and fixed in 70 percent ethanol. After at least four hours of fixation, the explanted tissues were x-rayed using a Micro-R x-ray cabinet, (20 keV x-rays, Polaroid Type 53 film, 20 second exposure time). The explanted tissue samples were embedded using polymerized glycol methacrylate (see Block, M.H., L. Trenner, P. Reugg, and M. Karr, "Glycol Methacrylate Embedding Technique", Laboratory Medicine, 13(5): 1982, pp. 290-298, which is incorporated herein by reference in its entirety), sectioned at a 4 micron thickness, stained with Toluidine Blue O or silver nitrate followed by hematoxylin and eosin, and evaluated histologically for osteogenesis and calcified tissue proliferation. Endochondral bone formation (as judged by explant mass measurements, x-ray evaluation and histologic evaluation) was readily demonstrated. The results are summarized in Table II below.

TABLE II

Purified OF Dose	Mass of Subcutaneous Explant (X ± SD mg)	X-Ray Score (See Fig. 2)	Histologic Grade (See Table IV)
Zero	NSF*	NSF	NSF
3.5 µg	75.5±4.9	3.0	3.0
10 µg	104.8±11.2	4.0	3.5
35 µg	109±9.1	4.0	4.0

* NSF = No Sample Found

PARTIALLY PURIFIED OSTEOINDUCTIVE FACTOR

[0064] Partially purified osteoinductive factor material was evaluated using these same procedures. A protein sample obtained following cation exchange chromatography but prior to HPLC purification was dissolved in 1 x 10^{-2} M hydrochloric acid at concentrations of 350, 100 and 3,500 µg of protein per ml HCl. One hundred µl aliquots of the above three test solutions were added to four collagen sponge discs for each test done. The solutions were allowed to soak into the collagen sponge discs for thirty minutes. The discs were then frozen at -50°C and lyophilized for about eighteen hours. These discs containing partially purified osteoinductive factor were implanted subcutaneously in rats. After 21 days the tissues were explanted and evaluated by the procedures described above. The results are summarized in Table III below.

TABLE III

Purified OF Dose	Mass of Subcutaneous Explant (X ± SD mg)	X-Ray Score (See Fig. 2)	Histologic Grade (See Table IV)
Zero	NSF*	NSF	NSF
35 µg	52.8±15.3	2.5	1.75
100 µg	100±14.1	3.5	3.25
350 µg	116±8.5	3.75	3.75

* NSF = No Sample Found

[0065] As can be appreciated by comparing Table II to Table III, the purified osteoinductive factor provides much greater osteoinductive activity at lower dosages than the partially purified osteoinductive factor. For this reason, the purified osteoinductive factor is preferred.

TABLE IV

SCORING CHARACTERISTICS FOR SUBCUTANEOUS IMPLANT BIOASSAY SAMPLES	
SCORE	CHARACTERISTIC HISTOLOGIC APPEARANCE
Zero (0)	- No residual implanted sample found, OR - section shows no silver staining deposits or those deposits are associated with acellular events, e. g., dystrophic mineralization of collagen fibrils. - Explants generally small, 2-4 mm diameter.
One (1)	- Tissue explant diameters generally smaller (3-5 mm) than original implants (7-8 mm). - Focal areas of silver staining mineralized tissues are of cellular origin. This may include mineralized cartilage as well as mineralized osteoid matrix. - Silver staining deposits randomly distribute within the explanted tissue section. - Silver stained areas typically encompass less than 50% of the total section area. - Explant centers are infiltrated with fibroblast-like cells, show little or no residual fibrillar collagen, and are not infarcted.
Two (2)	- Larger than grade 1 explants, but not as large as original implanted discs. - Silver staining areas generally localized in the outer regions of the explant. - Silver staining tissue is substantially all mineralized cartilage or dystrophic mineral radiating inward from mineralized cartilage showing little bone formation or few active osteoblastic surfaces. - Little or no hematopoietic bone marrow present. - Explant centers often show residual, non-resorbed fibrillar collagen sponge.
Three (3)	- Silver staining mineralized tissue clearly shows a circular pattern occurring near the outer regions of the explant. - Both mineralized cartilage and active osteoblast surfaces present. More mature bone forming closest to the periphery of the explant.

TABLE IV (continued)

SCORING CHARACTERISTICS FOR SUBCUTANEOUS IMPLANT BIOASSAY SAMPLES	
SCORE	CHARACTERISTIC HISTOLOGIC APPEARANCE
	- Hematopoietic bone marrow elements present. - Clear evidence for osteoclastic resorption of mineralized cartilage occurring. - Dystrophic mineralization often seen toward center of explant inside of the region of true bone formation. - Fewer cells present in center of explant than seen in Score 2 samples. Residual collagen sponge often evident. - Areas of chondrocyte proliferation often present but not extensive. - Blood capillaries invading mineralized area.
Four (4)	- Silver staining mineralized tissues clearly form as a thin rim of bone at the periphery of the section. - Most of the mineralized cartilage has been resorbed. - Bone clearly of osteoblastic origin, with many active osteoblast surfaces present. - Often large area of proliferating chondrocytes and extracellular matrix evident. - Hematopoietic bone marrow present. - The "pseudo-periosteum" is thin (≈ 10 cell layers) and composed primarily of cells which appear to be fibroblastic. - The center of the section often infarcted and largely acellular.

[0066] While various embodiments of the present invention have been described- in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. For example, the osteoinductive factors can be used in various applications such as treating periodontal diseases and in facial reconstruction, as well as in treating other bone and joint problems.

Claims

1. An osteoinductive mixture of proteins that is soluble in water comprising an amino acid composition of from 20.7 to 26.1 mole percent acidic amino acids, 11.3 to 15.7 mole percent hydroxy amino acids, 37.6 to 42.4 mole percent aliphatic amino acids, 5.8 to 7.9 mole percent aromatic amino acids and 13.3 to 19.9 mole percent basic amino acids, wherein use of said mixture of proteins results in a histological grade of at least about 3 when subcutaneously implanted for 21 days at a protein:substrate weight ratio of about 1:600.

2. The osteoinductive mixture of proteins, as claimed in Claim 1, comprising, upon hydrolysis, an amino acid composition of from 20.7 to 26.1 mole percent ASP(+ASN) and GLU(+GLN); from 11.3 to 15.7 mole percent SER and THR; from 37.6 to 42.4 mole percent ALA, GLY, PRO, MET, VAL, ILE, and LEU; from 5.8 to 7.9 mole percent TYR and PHE; and from 13.3 to 19.9 mole percent HIS, ARG, and LYS, based on the total moles of said amino acids.

3. The osteoinductive mixture of proteins, as claimed in Claim 1, which, when subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, results in a reduced or non-reduced gel profile comprising all of the protein

bands shown in Figure 1.

4. The osteoinductive mixture of proteins, as claimed in Claim 1, having the following amino acid mole percentages:

Amino Acid	Mole Percent
Asp	11.14
Glu	12.25
Ser	9.48
Gly	8.50
His	2.28
Arg	7.19
Thr	4.03
Ala	8.05
Pro	7.16
Tyr	3.63
Val	3.79
Met	1.73
Ile	2.75
Leu	8.00
Phe	3.21
Lys	7.11

5. An osteoinductive mixture of proteins that is soluble in water which, when subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, results in a reduced or non-reduced gel profile comprising all of the protein bands shown in Figure 1, wherein said mixture of proteins achieves a histological grade of at least about 3 when subcutaneously implanted for 21 days at a protein: substrate weight ratio of about 1:600.

6. The osteoinductive mixture of proteins, as claimed in claim 5, having, upon hydrolysis, an amino acid composition of from 20.7 to 26.1 mole percent ASP(+ASN) and GLU(+GLN); from 11.3 to 15.7 mole percent SER and THR; from 37.6 to 42.4 mole percent ALA, GLY, PRO, MET, VAL, ILE, and LEU; from 5.8 to 7.9 mole percent TYR and PHE; and from 13.3 to 19.9 mole percent HIS, ARG, and LYS, based on the total moles of said amino acids.

7. The osteoinductive mixture of proteins, as claimed in Claim 5, having the following amino acid mole percentages:

Amino Acid	Mole Percent
Asp	11.14
Glu	12.25
Ser	9.48
Gly	8.50
His	2.28
Arg	7.19
Thr	4.03
Ala	8.05
Pro	7.16
Tyr	3.63

(continued)

Amino Acid	Mole Percent
Val	3.79
Met	1.73
Ile	2.75
Leu	8.00
Phe	3.21
Lys	7.11

8. A process for purifying osteoinductively active proteins according to claim 1 from a first solution containing extract of demineralized bone, comprising:

(a) subjecting said first solution to ultrafiltration to obtain a second solution containing proteins having a desired range of molecular weights;
(b) loading said second solution onto an anion exchange resin;
(c) eluting proteins from said anion exchange resin with a first eluant to obtain an anion exchanged fraction eluate;
(d) loading said anion exchanged fraction eluate onto a cation exchange resin; and
(e) eluting proteins from said cation exchange resin with a second eluant to obtain a cation exchanged fraction eluate.

9. A process, as claimed in claim 8, further comprising the step of loading a third solution of proteins from said cation exchanged fraction onto a reverse phase HPLC column.

10. A process, as claimed in claim 8, wherein said ultrafiltration comprises:

(i) a first ultrafiltration step wherein said first solution is subjected to tangential flow ultrafiltration using an ultrafiltration membrane having a nominal MWCO of approximately 100 kD; and
(ii) subjecting the filtrate from said first ultrafiltration step to a second tangential flow ultrafiltration step using an ultrafiltration membrane having a nominal MWCO of approximately 10 kD and subjecting the retentate from this second ultrafiltration step to further processing.

11. A process, as claimed in claim 8, wherein the conductivity of said second solution from said ultrafiltration step is adjusted, if necessary, to a conductivity of less than about 1,900 μmhos (1.9×10^{-3} S) prior to loading onto said anion exchange resin.

12. A process, as claimed in Claim 8, wherein said first eluant has a conductivity in a range from about 10,260 μmhos (1.026×10^{-2} S) to about 11,200 μmhos (1.120×10^{-2} S).

13. A process, as claimed in Claim 8, wherein said anion exchanged fraction eluate is adjusted, if necessary, to a conductivity in a range from about 17,900 μmhos (1.79×10^{-2} S) to about 19,200 μmhos (1.92×10^{-2} S) prior to loading onto said cation exchange resin.

14. A process, as claimed in claim 8, wherein said second eluant has a conductivity from about 39,100 μmhos (3.91×10^{-2} S) to about 82,700 μmhos (8.27×10^{-2} S) or more.

15. A process, as claimed in claim 9, further comprising the step of eluting proteins from said HPLC column with a third eluant having a gradient of increasing acetonitrile concentration ranging from about 30 percent by volume to about 40 percent by volume to obtain a purified mixture comprising osteoinductively active proteins.

16. A process, as claimed in Claim 15, wherein said gradient of increasing acetonitrile concentration of said third eluant increases from about 33 percent by volume to about 37 percent by volume.

17. A process, as claimed in Claim 8, wherein said anion exchange resin comprises quaternary amine functional

groups.

18. A process, as claimed in claim 8, wherein said anion exchange resin comprises Q-Sepharose™ (Pharmacia).

19. A process, as claimed in claim 8, wherein said cation exchange resin comprises sulfonic acid functional groups.

20. A process, as claimed in claim 8, wherein said cation exchange resin comprises S-Sepharose™ (Pharmacia).

21. A process, as claimed in claim 9, wherein said HPLC column comprises either a plastic or hydrocarbon-modified silica packing.

22. A process, as claimed in Claim 21, wherein said silica is modified by the addition of C_4 through C_{18} hydrocarbons.

23. A process, as claimed in Claim 9, wherein said HPLC column comprises a packing material selected from the group consisting of VYDAC™ (The Separation Group) C_4, C_8 or C_{18} material.

24. A process, as claimed in claim 8, wherein the pH of said second solution is from about pH 8 to about pH 9 prior to said step of loading said second solution onto said anion exchange resin.

25. A process, as claimed in Claim 8, wherein the pH of said anion exchanged fraction eluate is from pH 4.4 to pH 5.0 prior to said step of loading said anion exchanged fraction eluate onto said cation exchange resin.

26. A process, as claimed in claim 8, wherein said second solution, said anion exchanged fraction eluate and said cation exchanged fraction eluate comprise an effective amount of urea to maintain proteins therein in solution.

27. A process, as claimed in Claim 8, wherein said second solution has a counterion concentration less than 0.135 M NaCl prior to loading onto said anion exchange resin.

28. A process, as claimed in claim 8, wherein said first eluant has a counterion concentration in a range from 0.23 M NaCl to 0.27 M NaCl.

29. A process, as claimed in Claim 8, wherein said second eluant has a counterion concentration in a range from 0.6 M NaCl to 1.5 M NaCl.

30. A process, as claimed in Claim 9, wherein said third solution comprises from 0.05 percent by volume to 0.15 percent by volume trifluoracetic acid.

31. A process, as claimed in claim 9, wherein said acetonitrile concentration in said third eluant is increased from about 33 percent by volume in increments of between 0.30 percent by volume and 0.40 percent by volume per minute until said concentration is about 37 percent by volume acetonitrile.

32. A process, as claimed in Claim 8, wherein said first solution containing demineralized bone extract is prepared by extracting proteins from cleaned, crushed demineralized bone particles using guanidine at a temperature of less than 20'C.

33. A process, as claimed in Claim 32, wherein urea is substituted for guanidine in a diafilter or dialysis process.

34. A process, as claimed in Claim 8, wherein an osteoinductive factor is produced having, upon hydrolysis, an amino acid composition of from 20.7 to 26.1 mole percent ASP(+ASN) and GLU(+GLN); from 11.3 to 15.7 mole percent SER and THR; from 37.6 to 42.4 mole percent ALA, GLY, PRO, MET, VAL, ILE, and LEU; from 5.8 to 7.9 mole percent TYR and PHE; and from 13.3 to 19.9 mole percent HIS, ARG, and LYS, wherein said mole percentages are based on the total moles of specified amino acids.

35. A process, as claimed in Claim 8 wherein a mixture of proteins is produced which, when subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, results in a reduced or non-reduced gel profile comprising all of the protein bands as shown in Figure 1.

36. A process for purifying osteoinductively active proteins comprising:

(a) crushing bone and cleaning said bone, including the step of removing soft tissue therefrom by flotation;
(b) demineralizing said crushed and cleaned bone particles using an acid;
(c) extracting proteins from the cleaned, crushed and demineralized bone particles using guanidine at low temperature to prevent the deterioration of said proteins and to obtain a first solution;
(d) subjecting said first solution to an ultrafiltration process comprising a first tangential flow ultrafiltration step employing an ultrafiltration membrane having a nominal MWCO of approximately 100 kD, retaining the filtrate and subjecting said filtrate to a second tangential flow ultrafiltration step employing an ultrafiltration membrane having a nominal MWCO of approximately 10 kD and retaining the retentate;
(e) substituting urea for said guanidine in said retentate by continuing the ultrafiltration step employing an ultrafiltration membrane having a nominal MWCO of approximately 10 kD while replacing guanidine lost in the filtrate with make-up urea to obtain an urea extract solution;
(f) adding NaCl to said urea extract solution to obtain a NaCl concentration of less than about 0.135 M and loading said urea extract solution onto a positive anion exchange resin having quaternary amine functional groups;
(g) eluting proteins from said positive anion exchange resin with a first eluant having a NaCl concentration of between about 0.23 M and about 0.27 M to obtain an anion exchanged fraction eluate;
(h) adjusting the pH of said anion exchanged fraction eluate to about pH 4.8;
(i) adding NaCl to said anion exchanged fraction eluate to obtain a NaCl concentration in a range from about 0.23 M to about 0.27 M and loading said anion exchanged fraction eluate onto a negative cation exchange resin having sulfonic acid functional groups;
(j) eluting proteins from said negative cation exchange resin with a second eluant having a NaCl concentration of between about 0.6 M and about 1.5 M to obtain a cation exchanged fraction eluate;
(k) dialyzing said cation exchanged fraction eluate to remove low molecular weight species;
(l) loading a second solution comprising proteins from said cation exchanged fraction onto a reverse phase HPLC column comprising hydrocarbon-modified silica packing material; and
(m) eluting proteins from said HPLC column with a third eluant, wherein said third eluant has a pH of less than about pH 2, a varying acetonitrile concentration increasing from about 33 percent by volume to about 37 percent by volume and a trifluoracetic acid concentration of between 0.05 percent by volume and 0.15 percent by volume.

37. A process, as claimed in claim 36, wherein said anion exchange resin is Q-Sepharose™ (Pharmacia).

38. A process, as claimed in Claim 36, wherein said cation exchange resin is S-Sepharose™ (Pharmacia).

39. A process, as claimed in claim 36, wherein said first solution, said anion exchanged fraction eluate and said cation exchanged fraction eluate each comprise an effective amount of urea to maintain proteins therein in solution.

40. A process, as claimed in Claim 36, wherein an osteoinductive factor is produced having, upon hydrolysis, an amino acid composition of from 20.7 to 26.1 mole percent ASP(+ASN) and GLU(+GLN); from 11.3 to 15.7 mole percent SER and THR; from 37.6 to 42.4 mole percent ALA, GLY, PRO, MET, VAL, ILE, and LEU; from 5.8 to 7.9 mole percent TYR and PHE; and from 13.3 to 19.9 mole percent HIS, ARG, and LYS, based on the total moles of said amino acids.

41. A process, as claimed in claim 36, wherein a mixture of proteins is produced which, when subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, results in a reduced or non-reduced gel profile comprising substantially all of the protein bands as shown in Figure 1.

42. An osteoinductive mixture of proteins that is soluble in water that achieves a histological grade of at least about 3 when subcutaneously implanted for 21 days at a protein:substrate weight ratio of about 1:600 produced by a process comprising:

(a) subjecting a solution containing demineralized bone extract to ultrafiltration;
(b) loading said solution onto an anion exchange resin;
(c) eluting proteins from said anion exchange resin to obtain an anion exchanged fraction eluate;
(d) loading said anion exchanged fraction eluate onto a strongly negative cation exchange resin;
(e) eluting proteins from said cation exchange resin to obtain a cation exchanged fraction eluate;
(f) loading a solution of proteins from said cation exchanged fraction onto a reverse phase HPLC column; and
(g) eluting proteins from said HPLC column with an eluant.

43. The osteoinductive mixture of proteins, as claimed in claim 42, wherein said process further comprises the step of loading a solution of proteins from said cation exchanged fraction onto a reverse phase HPLC column.

44. The osteoinductive mixture of proteins, as claimed in Claim 43, wherein said soluble mixture of proteins exhibits osteoinductive activity at about 3 micrograms when deposited onto a carrier suitable for induction of bone formation and implanted subcutaneously.

45. An osteoinductive mixture of proteins that is soluble in water that achieves a histological grade of at least about 3 when subcutaneously implanted for 21 days at a protein:substrate weight ratio of about 1:600 produced by a process comprising:

(a) subjecting a solution containing demineralized bone extract to ultrafiltration comprising a first ultrafiltration step employing an ultrafiltration membrane having a nominal MWCO of approximately 100 kD, retaining the filtrate and subjecting said filtrate to a second ultrafiltration step employing an ultrafiltration membrane having a nominal MWCO of approximately 10 kD and retaining the retentate;
(b) loading said retentate having a pH of pH 8.5 onto an anion exchange resin having quaternary amine functional groups, wherein said solution has a NaCl concentration of less than 0.135 M;
(c) eluting proteins from said anion exchange resin with an eluant having a NaCl concentration of between 0.23 M and 0.27 M to obtain an anion exchanged fraction;
(d) adjusting the pH of said anion exchanged fraction to pH 4.8;
(e) loading said anion exchanged fraction onto a cation exchange resin having sulfonic acid functional groups wherein said anion exchanged fraction has a NaCl concentration between 0.23 M and 0.27 M;
(f) eluting proteins from said cation exchange resin with an eluant having a NaCl concentration of between 0.6 M and 1.5 M to obtain a cation exchanged fraction;
(g) dialyzing said cation exchanged fraction eluate to remove low molecular weight species;
(h) loading proteins from said cation exchanged fraction onto a reverse phase HPLC column comprising hydrocarbon-modified silica packing material; and
(i) eluting proteins from said HPLC column with an eluant, wherein said eluant has a pH of less than about pH 2, an acetonitrile concentration ranging from about 33 percent by volume to about 37 percent by volume and a trifluoracetic acid concentration from 0.1 percent by volume to 0.15 percent by volume.

Patentansprüche

1. Wasserlösliche osteoinduktive Mischung von Proteinen, welche die folgende Aminosäure-Zusammensetzung aufweist:

20,7 bis 26,1 Mol-% saure Aminosäuren,
11,3 bis 15,7 Mol-% Hydroxyaminosäuren,
37,6 bis 42,4 Mol-% aliphatische Aminosäuren,
5,8 bis 7,9 Mol-% aromatische Aminosäuren und
13,3 bis 19,9 Mol-% basische Aminosäuren,

wobei die Verwendung der genannten Mischung von Proteinen zu einem histologischen Grad von mindestens etwa 3 führt, wenn sie 21 Tage lang subkutan implantiert wird bei einem Protein:Substrat-Gewichtsverhältnis von etwa 1:600.

2. Osteoinduktive Mischung von Proteinen nach Anspruch 1, die nach der Hydrolyse die folgende Aminosäure-Zusammensetzung aufweist:

20,7 bis 26,1 Mol-% ASP(+ASN) und GLU(+GLN);
11,3 bis 15,7 Mol-% SER und THR;
37,6 bis 42,4 Mol-% ALA, GLY, PRO, MET, VAL, ILE und LEU;
5,8 bis 7,9 Mol-% TYR und PHE; und
13,3 bis 19,9 Mol-% HIS, ARG und LYS,

bezogen auf die Gesamtanzahl der Mole der genannten Aminosäuren.

3. Osteoinduktive Mischung von Proteinen nach Anspruch 1, die, wenn sie einer Natriumdodecylsulfat-Polyacrylamid-Gel-Elektrophorese unterworfen wird, ein reduziertes oder nicht-reduziertes Gelprofil ergibt, das alle in Fig. 1 dargestellten Proteinbanden umfaßt.

4. Osteoinduktive Mischung von Proteinen nach Anspruch 1, welche die folgenden Molprozentsätze an Aminosäuren enthält:

Aminosäure	Mol-%
Asp	11,14
Glu	12,25
Ser	9,48
Gly	8,50
His	2,28
Arg	7,19
Thr	4,03
Ala	8,05
Pro	7,16
Tyr	3,63
Val	3,79
Met	1,73
Ile	2,75
Leu	8,00
Phe	3,21
Lys	7,11

5. Wasserlösliche osteoinduktive Mischung von Proteinen, die, wenn sie einer Natriumdodecylsulfat-Polyacrylamid-Gel-Elelektrophorese unterworfen wird, ein reduziertes oder nicht-reduziertes Gelprofil ergibt, das alle in Fig. 1 dargestellten Proteinbanden umfaßt, wobei die genannte Mischung von Proteinen einen histologischen Grad von mindestens etwa 3 ergibt, wenn sie 21 Tage lang subkutan implantiert wird bei einem Protein:Substrat-Gewichtsverhältnis von etwa 1:600.

6. Osteoinduktive Mischung von Proteinen nach Anspruch 5, die nach der Hydrolyse die folgende Aminosäure-Zusammensetzung aufweist:

20,7 bis 26,1 Mol-% ASP(+ASN) und GLU(+GLN);
11,3 bis 15,7 Mol-% SER und THR;
37,6 bis 42,4 Mol-% ALA, GLY, PRO, MET, VAL, ILE und LEU;
5,8 bis 7,9 Mol-% TYR und PHE; und
13,3 bis 19,9 Mol-% HIS, ARG und LYS,

bezogen auf die Gesamtanzahl der Mole der genannten Aminosäuren.

7. Osteoinduktive Mischung von Proteinen nach Anspruch 5, welche die folgenden Molprozentsätze an Aminosäuren enthält:

Aminosäure	Mol-%
Asp	11,14
Glu	12,25

(fortgesetzt)

Aminosäure	Mol-%
Ser	9,48
Gly	8,50
His	2,28
Arg	7,19
Thr	4,03
Ala	8,05
Pro	7,16
Tyr	3,63
Val	3,79
Met	1,73
Ile	2,75
Leu	8,00
Phe	3,21
Lys	7,11

8. Verfahren zur Reinigung der osteoinduktiv aktiven Proteine nach Anspruch 1 aus einer ersten Lösung, die einen Extrakt von entmineralisiertem Knochen enthält, wobei das Verfahren umfaßt:

(a) die Durchführung einer Ultrafiltration mit der genannten ersten Lösung unter Erzielung einer zweiten Lösung, die Proteine mit dem gewünschten Bereich der Molekulargewichte enthält;
(b) das Beladen eines Anionenaustauscherharzes mit der genannten zweiten Lösung;
(c) das Eluieren von Proteinen aus dem genannten Anionenaustauscherharz mit einem ersten Eluierungsmittel unter Erzielung eines Eluats aus einer Anionen-ausgetauschten Fraktion;
(d) das Beladen eines Kationenaustauscherharzes mit dem genannten Eluat aus der Anionen-ausgetauschten Fraktion; und
(e) das Eluieren von Proteinen aus dem genannten Kationenaustauscherharz mit einem zweiten Eluierungsmittel unter Erzielung eines Eluats aus einer Kationen-ausgetauschten Fraktion.

9. Verfahren nach Anspruch 8, das außerdem umfaßt das Beladen einer Umkehrphasen-HPLC-Kolonne mit einer dritten Lösung von Proteinen aus der genannten Kationen-ausgetauschten Fraktion.

10. Verfahren nach Anspruch 8, bei dem die genannte Ultrafiltration umfaßt:

(i) eine erste Ultrafiltrationsstufe, in der die genannte erste Lösung einer Tangentialstrom-Ultrafiltration unterworfen wird unter Verwendung einer Ultrafiltrationsmembran mit einem nominellen MWCO von etwa 100 kD; und
(ii) eine zweite Tangentialstrom-Ultrafiltrationsstufe, der das Filtrat aus der genannten ersten Ultrafiltrationsstufe unterworfen wird, unter Verwendung einer Ultrafiltrationsmembran mit einem nominellen MWCO von etwa 10 kD und das Weiterverarbeiten des Retentats aus dieser zweiten Ultrafiltrationsstufe.

11. Verfahren nach Anspruch 8, worin die elektrische Leitfähigkeit der genannten zweiten Lösung aus der genannten Ultrafiltrationsstufe erforderlichenfalls auf eine elektrische Leitfähigkeit von weniger als etwa 1 900 µmhos ($1{,}9 \times 10^{-3}$ S) eingestellt wird, bevor das genannte Anionenaustauscherharz mit dieser Lösung beladen wird.

12. Verfahren nach Anspruch 8, worin das genannte erste Eluat eine elektrische Leitfähigkeit in dem Bereich von etwa 10 260 µmhos ($1{,}026 \times 10^{-2}$ S) bis etwa 11 200 µmhos ($1{,}120 \times 10^{-2}$ S) aufweist.

13. Verfahren nach Anspruch 8, worin das genannte Eluat aus der Anionen-ausgetauschten Fraktion erforderlichen-

falls auf eine elektrische Leitfähigkeit in einem Bereich von etwa 17 900 µmhos ($1,79 \times 10^{-2}$ S) bis etwa 19 200 µmhos ($1,92 \times 10^{-2}$ S) eingestellt wird, bevor das genannte Kationenaustauscherharz mit dem Eluat beladen wird.

14. Verfahren nach Anspruch 8, worin das genannte zweite Eluat eine elektrische Leitfähigkeit von etwa 39 100 µmhos ($3,91 \times 10^{-2}$ S) bis etwa 82 700 µmhos ($8,27 \times 10^{-2}$ S) oder mehr aufweist.

15. Verfahren nach Anspruch 9, das außerdem umfaßt das Eluieren von Proteinen aus der genannten HPLC-Kolonne mit einem dritten Eluierungsmittel, das einen Gradienten einer steigenden Acetonitril-Konzentration in dem Bereich von etwa 30 Vol.-% bis etwa 40 Vol.-% aufweist, unter Erzielung einer gereinigten Mischung, die osteoinduktiv aktive Proteine umfaßt.

16. Verfahren nach Anspruch 15, worin der genannte Gradient der steigenden Acetonitril-Konzentration des genannten dritten Eluierungsmittels von etwa 33 Vol.-% auf etwa 37 Vol.-% ansteigt.

17. Verfahren nach Anspruch 8, worin das genannte Anionenaustauscherharz funktionelle quaternäre Amin-Gruppen umfaßt.

18. Verfahren nach Anspruch 8, worin das genannte Anionenaustauscherharz Q-Sepharose™ (Pharmacia) umfaßt.

19. Verfahren nach Anspruch 8, worin das genannte Kationenaustauscherharz funktionelle Sulfonsäuregruppen umfaßt.

20. Verfahren nach Anspruch 8, worin das genannte Kationenaustauscherharzes S-Sepharose™ (Pharmacia) umfaßt.

21. Verfahren nach Anspruch 9, worin die genannte HPLC-Kolonne entweder eine Kunststoff- oder Kohlenwasserstoff-modifizierte Siliciumdioxid-Füllung umfaßt.

22. Verfahren nach Anspruch 21, worin das genannte Siliciumdioxid durch Zugabe von C_4-C_{18}-Kohlenwasserstoffen modifiziert worden ist.

23. Verfahren nach Anspruch 9, worin die genannte HPLC-Kolonne ein Füllungsmaterial umfaßt, das ausgewählt wird aus der Gruppe, die besteht aus VYDAC™ (The Separation Group), C_4-, C_8- oder C_{18}-Material.

24. Verfahren nach Anspruch 8, worin der pH-Wert der genannten zweiten Lösung etwa 8 bis etwa 9 beträgt vor der Stufe des Beladens des genannten Anionenaustauscherharzes mit der genannten zweiten Lösung.

25. Verfahren nach Anspruch 8, worin der pH-Wert des genannten Eluats aus der Anionen-ausgetauschten Fraktion 4,4 bis 5,0 beträgt vor dem Beladen des genannten Kationenaustauscherharzes mi dem genannten Eluat aus der Anionen-ausgetauschten Fraktion.

26. Verfahren nach Anspruch 8, worin die genannte zweite Lösung, das genannte Eluat aus der Anionen-ausgetauschten Fraktion und das genannte Eluat aus der Kationen-ausgetauschten Fraktion eine wirksame Menge Harnstoff enthalten, um die Proteine darin in Lösung zu halten.

27. Verfahren nach Anspruch 8, worin die genannte zweite Lösung eine Gegenionen-Konzentration von weniger als 0,135 M NaCl aufweist vor dem Beladen des genannten Anionenaustauscherharzes mit dieser Lösung.

28. Verfahren nach Anspruch 8, worin das genannte erste Eluierungsmittel eine Gegenionen-Konzentration in einem Bereich von 0,23 bis 0,27 M NaCl aufweist.

29. Verfahren nach Anspruch 8, worin das genannte zweite Eluierungsmittel eine Gegenionen-Konzentration in einem Bereich von 0,6 bis 1,5 M NaCl aufweist.

30. Verfahren nach Anspruch 9, worin die genannte dritte Lösung 0,05 bis 0,15 Vol.-% Trifluoressigsäure umfaßt.

31. Verfahren nach Anspruch 9, worin die genannte Acetonitril-Konzentration in dem genannten dritten Eluierungsmittel von etwa 33 Vol.-% in Stufen zwischen 0,30 Vol.-% und 0,40 Vol.-% pro min bis zu der genannten Konzentration von etwa 37 Vol.-% Acetonitril erhöht wird.

32. Verfahren nach Anspruch 8, worin die genannte erste Lösung, die entmineralisierten Knochenextrakt enthält, hergestellt wird durch Extrahieren von Proteinen aus gereinigten, zerkleinerten, entmineralisierten Knochenteilchen unter Verwendung von Guanidin bei einer Temperatur von weniger als 20°C.

33. Verfahren nach Anspruch 32, worin das Guanidin durch Harnstoff ersetzt wird in einem Diafilter oder in einem Dialyseverfahren.

34. Verfahren nach Anspruch 8, bei dem ein osteoinduktiver Faktor gebildet wird, der nach der Hydrolyse die folgende Aminosäure-Zusammensetzung aufweist:

20,7 bis 26,1 Mol-% ASP(+ASN) und GLU(+GLN);
11,3 bis 15,7 Mol-% SER und THR;
37,6 bis 42,4 Mol-% ALA, GLY, PRO, MET, VAL, ILE und LEU;
5,8 bis 7,9 Mol-% TYR und PHE; und
13,3 bis 19,9 Mol-% HIS, ARG und LYS,

wobei die genannten Molprozentsätze auf die Gesamtanzahl der Mole der angegebenen Aminosäuren bezogen sind.

35. Verfahren nach Anspruch 8, bei dem eine Mischung von Proteinen gebildet wird, die nach der Durchführung einer Natriumdodecylsulfat-Polyacrylamid-Gel-Elektrophorese ein reduziertes oder nicht-reduziertes Gelprofil ergibt, das alle in Fig. 1 dargestellten Proteinbanden umfaßt.

36. Verfahren zur Reinigung von osteoinduktiv aktiven Proteinen, das umfaßt:

(a) das Zerkleinern eines Knochens und das Reinigen des genannten Knochens einschließlich der Entfernung des weichen Gewebes daraus durch Flotation;
(b) das Entmineralisieren der zerkleinerten und gereinigten Knochenteilchen unter Verwendung einer Säure;
(c) das Extrahieren von Proteinen aus den gereinigten, zerkleinerten und entmineralisierten Knochenteilchen unter Verwendung von Guanidin bei einer niedrigen Temperatur, um einen Abbau der genannten Proteine zu verhindern, unter Erzielung einer ersten Lösung;
(d) die Durchführung eines ersten Ultrafiltrationsprozesses mit der genannten ersten Lösung, der umfaßt eine erste Tangentialstrom-Ultrafiltrationsstufe, in der eine Ultrafiltrationsmembran mit einem nominellen MWCO von etwa 100 kD verwendet wird, das Sammeln des Filtrats und die Durchführung einer zweiten Tangentialstrom-Ultrafiltrationsstufe mit dem genannten Filtrat unter Verwendung einer Ultrafiltrationsmembran mit einem nominellen MWCO von etwa 10 kD und das Sammeln des Retentats;
(e) das Ersetzen des genannten Guanidins in dem genannten Retentat durch Harnstoff unter Fortsetzung der Ultrafiltrationsstufe, in der eine Ultrafiltrationsmembran mit einem nominellen MWCO von etwa 10 kD verwendet wird, während das in dem Filtrat verloren gegangene Guanidin durch Ergänzungs-Harnstoff ersetzt wird unter Erzielung einer Harnstoffextrakt-Lösung;
(f) die Zugabe von NaCl zu der genannten Harnstoffextrakt-Lösung unter Erzielung einer NaCl-Konzentration von weniger als etwa 0,135 M und das Beladen eines positiven Anionenaustauscherharzes, das funktionelle quaternäre Amin-Gruppen aufweist, mit der genannten Harnstoffextrakt-Lösung;
(g) das Eluieren von Proteinen aus dem genannten positiven Anionenaustauscherharz mit einem ersten Eluierungsmittel, das eine NaCl-Konzentration zwischen etwa 0,23 M und etwa 0,27 M aufweist, unter Erzielung eines Eluats aus einer Anionen-ausgetauschten Fraktion;
(h) das Einstellen des pH-Wertes des genannten Eluats aus der Anionen-ausgetauschten Fraktion auf etwa 4,8;
(i) die Zugabe von NaCl zu dem genannten Eluat aus der Anionen-ausgetauschten Fraktion unter Erzielung einer NaCl-Konzentration in einem Bereich von etwa 0,23 bis etwa 0,27 M und das Beladen eines negativen Kationenaustauscherharzes, das funktionelle Sulfonsäuregruppen aufweist, mit dem genannten Eluat aus der Anionen-ausgetauschten Fraktion;
(j) das Eluieren von Proteinen aus dem genannten negativen Kationenaustauscherharz mit einem zweiten Eluierungsmittel, das eine NaCl-Konzentration zwischen etwa 0,6 und etwa 1,5 M aufweist, unter Erzielung eines Eluats aus einer Kationen-ausgetauschten Fraktion;
(k) das Dialysieren des genannten Eluats aus der Kationen-ausgetauschten Fraktion zur Entfernung von Species mit niedrigem Molekulargewicht;
(l) das Beladen einer Umkehrphasen-HPLC-Kolonne, die ein mit Kohlenwasserstoff modifiziertes Siliciumdi-

oxid-Füllungsmaterial enthält, mit einer zweiten Lösung, die Proteine aus der genannten Kationen-ausgetauschten Fraktion enthält; und
(m) das Eluieren von Proteinen aus der genannten HPLC-Kolonne mit einem dritten Eluierungsmittel, wobei das genannte dritte Eluierungsmittel einen pH-Wert von weniger als etwa 2, eine variierende Acetonitril-Konzentration, die von etwa 33 Vol.-% auf etwa 37 Vol.-% ansteigt, und eine Trifluoressigsäure-Konzentration zwischen 0,05 Vol.-% und 0,15 Vol.-% aufweist.

37. Verfahren nach Anspruch 36, worin das genannte Anionenaustauscherharz Q-Sepharose™ (Pharmacia) ist.

38. Verfahren nach Anspruch 36, worin das genannte Kationenaustauscherharz S-Sepharose™ (Pharmacia) ist.

39. Verfahren nach Anspruch 36, worin die genannte ersten Lösung, das genannte Eluat aus der Anionen-ausgetauschten Fraktion und das genannte Eluat aus der Kationen-ausgetauschten Fraktion jeweils eine wirksame Menge Harnstoff enthalten, um die Proteine darin in Lösung zu halten.

40. Verfahren nach Anspruch 36, bei dem ein osteoinduktiver Faktor gebildet wird, der nach der Hydrolyse die folgende Aminosäure-Zusammensetzung aufweist:

20,7 bis 26,1 Mol-% ASP(+ASN) und GLU(+GLN);
11,3 bis 15,7 Mol-% SER und THR;
37,6 bis 42,4 Mol-% ALA, GLY, PRO, MET, VAL, ILE und LEU;
5,8 bis 7,9 Mol-% TYR und PHE; und
13,3 bis 19,9 Mol-% HIS, ARG und LYS,

bezogen auf die Gesamtanzahl der Mole der genannten Aminosäuren.

41. Verfahren nach Anspruch 36, bei dem eine Mischung von Proteinen gebildet wird, die nach der Durchführung einer Natriumdodecylsulfat-Polyacrylamid-Gel-Elektrophorese damit ein reduziertes oder nicht-reduziertes Gelprofil ergibt, das im wesentlichen alle in Fig. 1 dargestellten Proteinbanden umfaßt.

42. Wasserlösliche osteoinduktive Mischung von Proteinen, die einen histologischen Grad von mindestens etwa 3 ergibt, wenn sie 21 Tage lang subkutan implantiert wird bei einem Protein:Substrat-Gewichtsverhältnis von etwa 1:600, die hergestellt worden ist nach einem Verfahren, das umfaßt:

(a) die Durchführung einer Ultrafiltration mit einer entmineralisierten Knochenextrakt enthaltenden Lösung;
(b) das Beladen eines Anionenaustauscherharzes mit der genannten Lösung;
(c) das Eluieren von Proteinen aus dem genannten Anionenaustauscherharz unter Erzielung eines Eluats aus einer Anionen-ausgetauschten Fraktion;
(d) das Beladen eines stark negativen Kationenaustauscherharzes mit dem genannten Eluat aus der Anionen-ausgetauschten Fraktion;
(e) das Eluieren von Proteinen aus dem genannten Kationenaustauscherharz unter Erzielung eines Eluats aus einer Kationen-ausgetauschten Fraktion;
(f) das Beladen einer Umkehrphasen-HPLC-Kolonne mit einer Lösung von Proteinen aus der genannten Kationen-ausgetauschten Fraktion; und
(g) das Eluieren von Proteinen aus der genannten HPLC-Kolonne mit einem Eluierungsmittel.

43. Osteoinduktive Mischung von Proteinen nach Anspruch 42, bei dem das genannte Verfahren außerdem umfaßt das Beladen einer Umkehrphasen-HPLC-Kolonne mit einer Lösung von Proteinen aus der genannten Kationen-ausgetauschten Fraktion.

44. Osteoinduktive Mischung von Proteinen nach Anspruch 43, in der die genannte lösliche Mischung von Proteinen eine osteoinduktive Aktivität von etwa 3 µg aufweist, wenn sie auf einem für die Induktion der Knochenbildung geeigneten Träger abgeschieden und subkutan implantiert wird.

45. Wasserlösliche osteoinduktive Mischung von Proteinen, die einen histologischen Grad von mindestens etwa 3 ergibt, wenn sie 21 Tage lang subkutan implantiert wird bei einem Protein:Substrat-Gewichtsverhältnis von etwa 1:600, die nach einem Verfahren hergestellt worden ist, das umfaßt:

(a) die Durchführung einer Ultrafiltration mit einer entmineralisierten Knochenextrakt enthaltenden Lösung, die umfaßt eine erste Ultrafiltrationsstufe unter Verwendung einer Ultrafiltrationsmembran mit einem nominellen MWCO von etwa 100 kD, das Sammeln des Filtrats und die Durchführung einer zweiten Ultrafiltrationsstufe mit dem genannten Filtrat unter Verwendung einer Ultrafiltrationsmembran mit einem nominellen MWCO von etwa 10 kD und das Sammeln des Retentats;
(b) das Beladen eines Anionenaustauscherharzes, das funktionelle quaternäre Amin-Gruppen aufweist, mit dem genannten Retentat, das einen pH-Wert von 8,5 aufweist, wobei die genannte Lösung eine NaCl-Konzentration von weniger als 0,135 M aufweist;
(c) das Eluieren von Proteinen aus dem genannten Anionenaustauscherharz mit einem Eluierungsmittel, das eine NaCl-Konzentration zwischen 0,23 und 0,27 M aufweist, unter Erzielung einer Anionen-ausgetauschten Fraktion;
(d) das Einstellen des pH-Wertes der genannten Anionen-ausgetauschten Fraktion auf 4,8;
(e) das Beladen eines Kationenaustauscherharzes, das funktionelle Sulfonsäure-Gruppen aufweist, mit der genannten Anionen-ausgetauschten Fraktion, die eine NaCl-Konzentration zwischen 0,23 und 0,27 M aufweist;
(f) das Eluieren von Proteinen aus dem genannten Kationenaustauscherharz mit einem Eluierungsmittel, das eine NaCl-Konzentration zwischen 0,6 und 1,5 M aufweist, unter Erzielung einer Kationen-ausgetauschten Fraktion;
(g) das Dialysieren des genannten Eluats aus der Kationen-ausgetauschten Fraktion zur Entfernung von Species mit niedrigem Molekulargewicht;
(h) das Beladen einer Umkehrphasen-HPLC-Kolonne, die ein Kohlenwasserstoff-modifiziertes Siliciumdioxid-Füllungsmaterial enthält, mit Proteinen aus der genannten Kationen-ausgetauschten Fraktion; und
(i) das Eluieren von Proteinen aus der genannten HPLC-Kolonne mit einem Eluierungsmittel, das einen pH-Wert von weniger als etwa 2, eine Acetonitril-Konzentration in dem Bereich von etwa 33 Vol.-% bis etwa 37 Vol.-% und eine Trifluoressigsäure-Konzentration von 0,1 Vol.-% bis 0,15 Vol.-% aufweist.

Revendications

1. Mélange de protéines ostéo-inductif qui est soluble dans l'eau, comprenant la composition d'acides aminés suivante : de 20,7 à 26,1 % en moles d'acides aminés acides, de 11,3 à 15,7 % en moles d'hydroxyacides aminés, de 37,6 à 42,4 % en moles d'acides aminés aliphatiques, de 5,8 à 7,9 % en moles d'acides aminés aromatiques et de 13,3 à 19,9 % en moles d'acides aminés basiques, dans lequel l'utilisation dudit mélange de protéines conduit à un grade histologique d'au moins environ 3 quand il est implanté par voie sous-cutanée pendant 21 jours à un rapport en poids protéine/substrat d'environ 1/600.

2. Mélange ostéo-inductif de protéines selon la revendication 1, comprenant, après hydrolyse, la composition d'acides aminés suivante : de 20,7 à 26,1 % en moles de ASP(+ASN) et GLU(+GLN) ; de 11,3 à 15,7 % en moles de SER et THR ; de 37,6 à 42,4 % en moles de ALA, GLY, PRO, MET, VAL, ILE et LEU ; de 5,8 à 7,9 % en moles de TYR et PHE ; et de 13,3 à 19,9 % en moles de HIS, ARG et LYS, par rapport au nombre total de moles desdits acides aminés.

3. Mélange ostéo-inductif de protéines selon la revendication 1, qui, lorsqu'il est soumis à une électrophorèse sur gel de polyacrylamide/dodécylsulfate de sodium, conduit à un profil de gel réduit ou non réduit comprenant toutes les bandes de protéine représentées sur la Figure 1.

4. Mélange ostéo-inductif de protéines selon la revendication 1, ayant les pourcentages molaires suivants d'acides aminés :

Acide aminé	Pourcentage en moles
Asp	11,14
Glu	12,25
Ser	9,48
Gly	8,50
His	2,28
Arg	7,19

(suite)

Acide aminé	Pourcentage en moles
Thr	4,03
Ala	8,05
Pro	7,16
Tyr	3,63
Val	3,79
Met	1,73
Ile	2,75
Leu	8,00
Phe	3,21
Lys	7,11

5. Mélange ostéo-inductif de protéines qui est soluble dans l'eau et qui, lorsqu'il est soumis à une électrophorèse sur gel de polyacrylamide/dodécylsulfate de sodium, conduit à un profil de gel réduit ou non réduit comprenant toutes les bandes de protéines représentées sur la Figure 1, dans lequel ledit mélange de protéines atteint un grade histologique d'au moins environ 3 quand il est implanté par voie sous-cutanée pendant 21 jours à un rapport en poids protéine/substrat d'environ 1/600.

6. Mélange ostéo-inductif de protéines selon la revendication 5, comprenant, après hydrolyse, la composition d'acides aminés suivante : de 20,7 à 26,1 % en moles de ASP(+ASN) et GLU(+GLN) ; de 11,3 à 15,7 % en moles de SER et THR ; de 37,6 à 42,4 % en moles de ALA, GLY, PRO, MET, VAL, ILE et LEU ; de 5,8 à 7,9 % en moles de TYR et PHE ; et de 13,3 à 19,9 % en moles de HIS, ARG et LYS, par rapport au nombre total de moles desdits acides aminés.

7. Mélange ostéo-inductif de protéines selon la revendication 5, ayant les pourcentages molaires suivants d'acides aminés :

Acide aminé	Pourcentage en moles
Asp	11,14
Glu	12,25
Ser	9,48
Gly	8,50
His	2,28
Arg	7,19
Thr	4,03
Ala	8,05
Pro	7,16
Tyr	3,63
Val	3,79
Met	1,73
Ile	2,75
Leu	8,00
Phe	3,21
Lys	7,11

8. Procédé pour purifier des protéines à action ostéo-inductive selon la revendication 1 à partir d'une première solution contenant de l'extrait d'os déminéralisé, consistant à :

(a) soumettre ladite première solution à une ultrafiltration pour obtenir une deuxième solution contenant des protéines ayant une plage souhaitée de poids moléculaires ;
(b) charger ladite deuxième solution sur une résine échangeuse d'anions ;
(c) éluer des protéines à partir de ladite résine échangeuse d'anions avec un premier éluant pour obtenir un

éluat de fraction ayant subi un échange d'anions ;
(d) charger ledit éluat de fraction ayant subi un échange d'anions sur une résine échangeuse de cations ; et
(e) éluer des protéines à partir de ladite résine échangeuse de cations avec un deuxième éluant pour obtenir un éluat de fraction ayant subi un échange de cations.

9. Procédé selon la revendication 8, comprenant en outre l'étape consistant à introduire une troisième solution de protéines venant de ladite fraction ayant subi un échange de cations dans une colonne de CLHP à inversion de phases.

10. Procédé selon la revendication 8, dans lequel ladite ultrafiltration comprend :

(i) une première étape d'ultrafiltration dans laquelle ladite première solution est soumise à une ultrafiltration par écoulement tangentiel utilisant une membrane d'ultrafiltration ayant une séparation de poids moléculaire nominale à environ 100 kD ; et
(ii) le fait de soumettre le filtrat provenant de ladite première étape d'ultrafiltration à une deuxième étape d'ultrafiltration par écoulement tangentiel utilisant une membrane d'ultrafiltration ayant une séparation de poids moléculaire nominale à environ 10 kD et de soumettre le rétentat provenant de cette deuxième étape d'ultrafiltration à un traitement ultérieur.

11. Procédé selon la revendication 8, dans lequel la conductivité de ladite deuxième solution provenant de ladite étape d'ultrafiltration est ajustée, si nécessaire, à une conductivité inférieure à environ 1 900 µmhos ($1{,}9 \times 10^{-3}$ S) avant chargement sur ladite résine échangeuse d'anions.

12. Procédé selon la revendication 8, dans lequel ledit premier éluant a une conductivité située dans la plage allant d'environ 10 260 µmhos ($1{,}026 \times 10^{-2}$ S) à environ 11 200 µmhos ($1{,}120 \times 10^{-2}$ S).

13. Procédé selon la revendication 8, dans lequel ledit éluat de fraction ayant subi un échange d'anions est ajusté, si nécessaire, à une conductivité située dans la plage allant d'environ 17 900 µmhos ($1{,}79 \times 10^{-2}$ S) à environ 19 200 µmhos ($1{,}92 \times 10^{-2}$ S) avec chargement sur ladite résine échangeuse de cations.

14. Procédé selon la revendication 8, dans lequel ledit deuxième éluant a une conductivité d'environ 39 100 µmhos ($3{,}91 \times 10^{-2}$ S) à environ 82 700 µmhos ($8{,}27 \times 10^{-2}$ S) ou plus.

15. Procédé selon la revendication 9, comprenant en outre l'étape d'élution de protéines à partir de ladite colonne de CLHP avec un troisième éluant ayant un gradient de concentration croissante d'acétonitrile allant d'environ 30 % en volume à environ 40 % en volume pour obtenir un mélange purifié comprenant des protéines à activité ostéoinductive.

16. Procédé selon la revendication 15, dans lequel ledit gradient de concentration croissante d'acétonitrile dudit troisième éluant augmente d'environ 33 % en volume à environ 37 % en volume.

17. Procédé selon la revendication 8, dans lequel ladite résine échangeuse d'anions comprend des groupes fonctionnels de type amine quaternaire.

18. Procédé selon la revendication 8, dans lequel ladite résine échangeuse d'anions comprend de la Q-Sepharose® (Pharmacia).

19. Procédé selon la revendication 8, dans lequel ladite résine échangeuse de cations comprend des groupes fonctionnels de type acide sulfonique.

20. Procédé selon la revendication 8, dans lequel ladite résine échangeuse de cations comprend de la S-Sepharose® (Pharmacia).

21. Procédé selon la revendication 9, dans lequel ladite colonne de CLHP comprend une garniture soit de matière plastique soit de silice modifiée par des hydrocarbures.

22. Procédé selon la revendication 21, dans lequel ladite silice est modifiée par l'addition d'hydrocarbures en C_4 à C_{18}.

23. Procédé selon la revendication 9, dans lequel ladite colonne de CLHP comprend un matériau de garniture choisi dans le groupe constitué par les matériaux VYDAC® (The Separation Group) en C_4, C_8 ou C_{18}.

24. Procédé selon la revendication 8, dans lequel le pH de ladite deuxième solution va d'environ 8 à environ 9 avant ladite étape de chargement de ladite deuxième solution sur ladite résine échangeuse d'anions.

25. Procédé selon la revendication 8, dans lequel le pH dudit éluat de fraction ayant subi un échange d'anions va de 4,4 à 5,0 avant ladite étape de chargement dudit éluat de fraction ayant subi un échange d'anions sur ladite résine échangeuse de cations.

26. Procédé selon la revendication 8, dans lequel ladite deuxième solution, ledit deuxième éluat de fraction ayant subi un échange d'anions, et ledit éluat de fraction ayant subi un échange de cations comprennent une quantité d'urée efficace pour maintenir les protéines en solution dans ceux-ci.

27. Procédé selon la revendication 8, dans lequel ladite deuxième solution a une concentration de contre-ions inférieure à 0,135 M de NaCl avant chargement sur ladite résine échangeuse d'anions.

28. Procédé selon la revendication 8, dans lequel ledit premier éluant a une concentration de contre-ions située dans la plage allant de 0,23 M de NaCl à 0,27 M de NaCl.

29. Procédé selon la revendication 8, dans lequel ledit deuxième éluant a une concentration de contre-ions située dans la plage allant de 0,6 M de NaCl à 1,5 M de NaCl.

30. Procédé selon la revendication 9, dans lequel ladite troisième composition comprend de 0,05 % en volume à 0,15 % en volume d'acide trifluoroacétique.

31. Procédé selon la revendication 9, dans lequel ladite concentration d'acétonitrile dans ledit troisième éluant est augmentée à partir d'environ 33 % en volume par incréments de 0,30 % en volume à 0,40 % en volume par minute jusqu'à ce que ladite concentration soit d'environ 37 % en volume d'acétonitrile.

32. Procédé selon la revendication 8, dans lequel ladite première solution contenant de l'extrait d'os déminéralisé est préparée par extraction de protéines à partir de particules d'os déminéralisés broyées nettoyées, par utilisation de guanidine à une température inférieure à 20°C.

33. Procédé selon la revendication 32, dans lequel l'urée remplace la guanidine dans un processus de diafiltration ou de dialyse.

34. Procédé selon la revendication 8, dans lequel un facteur ostéo-inductif est produit, lequel a, après hydrolyse, la composition d'acides aminés suivante : de 20,7 à 26,1 % en moles de ASP(+ASN) et GLU(+GLN) ; de 11,3 à 15,7 % en moles de SER et THR ; de 37,6 à 42,4 % en moles de ALA, GLY, PRO, MET, VAL, ILE et LEU ; de 5,8 à 7,9 % en moles de TYR et PHE ; et de 13,3 à 19,9 % en moles de HIS, ARG et LYS, où lesdits pourcentages en moles sont basés sur le nombre total de moles desdits acides aminés spécifiés.

35. Procédé selon la revendication 8, dans lequel un mélange de protéines est produit, lequel, lorsqu'il est soumis à une électrophorèse sur gel de polyacrylamide/dodécylsulfate de sodium, conduit à un profil de gel réduit ou non réduit comprenant toutes les bandes de protéine représentées sur la Figure 1.

36. Procédé pour purifier des protéines à activité ostéo-inductive, consistant à :

(a) broyer de l'os et nettoyer ledit os, y compris l'étape d'élimination des tissus mous à partir de ceux-ci par flottation ;
(b) déminéraliser lesdites particules d'os broyé et nettoyé en utilisant un acide ;
(c) extraire des protéines à partir des particules d'os nettoyé, broyé et déminéralisé, en utilisant de la guanidine à basse température pour empêcher la détérioration desdites protéines et pour obtenir une première solution ;
(d) soumettre ladite première solution à un processus d'ultrafiltration comprenant une première étape d'ultrafiltration par écoulement tangentiel employant une membrane d'ultrafiltration ayant une séparation de poids moléculaire nominale à environ 100 kD, la conservation du filtrat, le fait de soumettre ledit filtrat à une deuxième étape d'ultrafiltration par écoulement tangentiel employant une membrane d'ultrafiltration ayant une séparation

de poids moléculaire nominale à environ 10 kD et la conservation du rétentat ;
(e) remplacer ladite guanidine par de l'urée dans ledit rétentat en poursuivant l'étape d'ultrafiltration employant une membrane d'ultrafiltration ayant une séparation de poids moléculaire nominale à environ 10 kD tout en remplaçant la guanidine perdue dans le filtrat avec de l'urée d'appoint pour obtenir une solution d'extrait d'urée ;
(f) ajouter du NaCl à ladite solution d'extrait d'urée pour obtenir une concentration de NaCl inférieure à environ 0,135 M et charger ladite solution d'extrait d'urée sur une résine échangeuse d'anions positive ayant des groupes fonctionnels de type amine quaternaire ;
(g) éluer des protéines à partir de ladite première résine échangeuse d'anions positive avec un premier éluant ayant une concentration de NaCl entre environ 0,23 M et environ 0,27 M pour obtenir un éluat de fraction ayant subi un échange d'anions ;
(h) ajuster le pH dudit éluat de fraction ayant subi un échange d'anions à environ 4,8 ;
(i) ajouter du NaCl audit éluat de fraction ayant subi un échange d'anions pour obtenir une concentration de NaCl située dans la plage allant d'environ 0,23 M à environ 0,27 M et charger ledit éluat de fraction ayant subi un échange d'anions sur une résine échangeuse de cations négative ayant des groupes fonctionnels de type acide sulfonique ;
(j) éluer des protéines à partir de ladite résine échangeuse de cations négative avec un deuxième éluant ayant une concentration de NaCl entre environ 0,6 M et environ 1,5 M pour obtenir un éluat de fraction ayant subi un échange de cations ;
(k) dialyser ledit éluat de fraction ayant subi un échange de cations pour éliminer les espèces de faible poids moléculaire ;
(l) introduire une deuxième solution comprenant des protéines provenant de ladite fraction ayant subi un échange de cations dans une colonne de CLHP à inversion de phases comprenant un matériau de garniture en silice modifiée par des hydrocarbures ; et
(m) éluer des protéines à partir de ladite colonne de CLHP avec un troisième éluant, où ledit troisième éluant a un pH inférieur à environ 2, une concentration d'acétonitrile variable augmentant d'environ 33 % en volume à environ 37 % en volume et une concentration d'acide trifluoroacétique de 0,05 % en volume à 0,15 % en volume.

37. Procédé selon la revendication 36, dans lequel ladite résine échangeuse d'anions est de la Q-Sepharose® (Pharmacia).

38. Procédé selon la revendication 36, dans lequel ladite résine échangeuse de cations est de la S-Sepharose® (Pharmacia).

39. Procédé selon la revendication 36, dans lequel ladite première solution, ledit éluat de fraction ayant subi un échange d'anions et ledit éluat de fraction ayant subi un échange de cations contiennent chacun une quantité d'urée efficace pour maintenir les protéines en solution dans ceux-ci.

40. Procédé selon la revendication 36, dans lequel un facteur ostéo-inductif est produit, lequel a, après hydrolyse, la composition d'acides aminés suivante : de 20,7 à 26,1 % en moles de ASP(+ASN) et GLU(+GLN) ; de 11,3 à 15,7 % en moles de SER et THR ; de 37,6 à 42,4 % en moles de ALA, GLY, PRO, MET, VAL, ILE et LEU ; de 5,8 à 7,9 % en moles de TYR et PHE ; et de 13,3 à 19,9 % en moles de HIS, ARG et LYS, par rapport au nombre total de moles desdits acides aminés.

41. Procédé selon la revendication 36, dans lequel un mélange de protéines est produit, lequel, lorsqu'il est soumis à une électrophorèse sur gel de polyacrylamide/dodécylsulfate de sodium, conduit à un profil de gel réduit ou non réduit comprenant toutes les bandes de protéine représentées sur la Figure 1.

42. Mélange ostéo-inductif de protéines qui est soluble dans l'eau, qui atteint un grade histologique d'au moins environ 3 quand il est implanté par voie sous-cutanée pendant 21 jours à un rapport en poids protéine/substrat d'environ 1/600, produit par un procédé consistant à :

(a) soumettre une solution contenant de l'extrait d'os déminéralisé à une ultrafiltration ;
(b) charger ladite solution sur une résine échangeuse d'anions ;
(c) éluer des protéines à partir de ladite résine échangeuse d'anions pour obtenir un éluat de fraction ayant subi un échange d'anions ;
(d) charger ledit éluat de fraction ayant subi un échange d'anions sur une résine échangeuse de cations fortement négative ;

(e) éluer des protéines à partir de ladite résine échangeuse de cations pour obtenir un éluat de fraction ayant subi un échange de cations ;
(f) introduire une solution de protéines provenant de ladite fraction ayant subi un échange de cations dans une colonne de CLPH à inversion de phases ; et
(g) éluer des protéines à partir de ladite colonne de CLPH avec un éluant.

43. Mélange ostéo-inductif de protéines selon la revendication 42, dans lequel ledit procédé comprend en outre l'étape consistant à introduire une solution de protéines provenant de ladite fraction ayant subi un échange de cations dans une colonne de CLPH à inversion de phases.

44. Mélange ostéo-inductif de protéines selon la revendication 43, dans lequel ledit mélange soluble de protéines présente une activité ostéo-inductive à environ 3 microgrammes quand il est déposé sur un support convenable pour l'induction de la formation osseuse et implanté par voie sous-cutanée.

45. Mélange ostéo-inductif de protéines qui est soluble dans l'eau, qui atteint un grade histologique d'au moins environ 3 quand il est implanté par voie sous-cutanée pendant 21 jours à un rapport en poids protéine/substrat d'environ 1/600, produit par un procédé consistant à :

(a) soumettre une solution contenant de l'extrait d'os déminéralisé à une ultrafiltration comprenant une première étape d'ultrafiltration employant une membrane d'ultrafiltration ayant une séparation de poids moléculaire nominale à environ 100 kD, la conservation du filtrat, le fait de soumettre ledit filtrat à une deuxième étape d'ultrafiltration par écoulement tangentiel employant une membrane d'ultrafiltration ayant une séparation de poids moléculaire nominale à environ 10 kD, et la conservation du rétentat ;
(b) charger ledit rétentat ayant un pH d'environ 8,5 sur une résine échangeuse d'anions ayant des groupes fonctionnels de type amine quaternaire, où ladite solution a une concentration de NaCl inférieure à 0,135 M ;
(c) éluer des protéines à partir de ladite résine échangeuse d'anions avec un éluant ayant une concentration de NaCl entre 0,23 M et 0,27 M pour obtenir une fraction ayant subi un échange d'anions ;
(d) ajuster le pH de ladite fraction ayant subi un échange d'anions à 4,8 ;
(e) charger ladite fraction ayant subi un échange d'anions sur une résine échangeuse de cations ayant des groupes fonctionnels de type acide sulfonique, où ladite fraction ayant subi un échange d'anions a une concentration de NaCl entre 0,23 M et 0,27 M ;
(f) éluer des protéines à partir de ladite résine échangeuse de cations avec un éluant ayant une concentration de NaCl entre 0,6 M et 1,5 M pour obtenir une fraction ayant subi un échange de cations ;
(g) dialyser ledit éluat de fraction ayant subi un échange de cations pour éliminer les espèces de faible poids moléculaire ;
(h) introduire des protéines provenant de ladite fraction ayant subi un échange de cations dans une colonne de CLPH à inversion de phases comprenant un matériau de garniture en silice modifiée par des hydrocarbures ; et
(i) éluer des protéines à partir de ladite colonne de CLPH avec un éluant, où ledit éluant a un pH inférieur à environ 2, une concentration d'acétonitrile située dans la plage allant d'environ 33 % en volume à environ 37 % en volume et une concentration d'acide trifluoroacétique d'environ 0,1 % en volume à environ 0,15 % en volume.

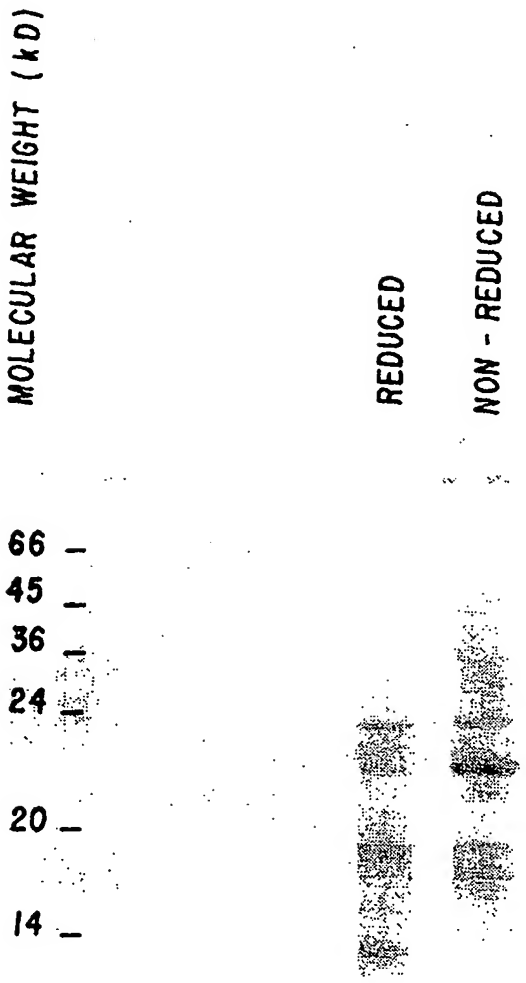

FIG. 1

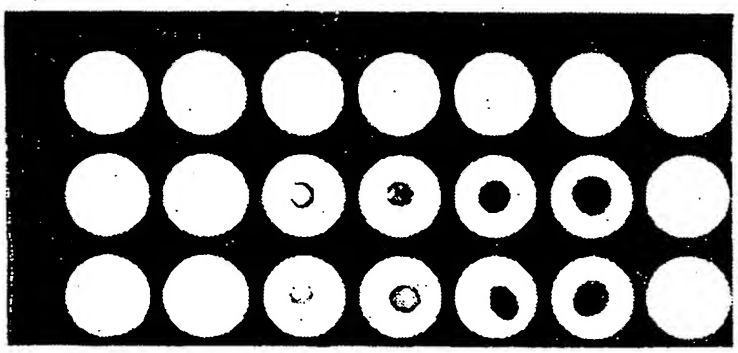

FIG. 2